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Nuclear localization of SYT, SSX and the synovial sarcoma-associated SYT–SSX fusion proteins

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Synovial sarcoma is characterized by a prevalent chromosomal translocation, t(X;18)(p11;q11). As a result of this translocation the SYT gene on chromosome 18 fuses to either the SSX1 or the SSX2 gene on the X chromosome. In this study, we generated polyclonal antibodies against the SYT and SSX2 proteins. These antibodies specifically detected both these proteins and the SYT–SSX fusion proteins in transfected COS-1 cell extracts. Indirect immunofluorescence analysis of COS-1 cells expressing tagged or untagged SYT, SSX2, SYT–SSX1 or SYT–SSX2 indicated that all these proteins are localized in the nucleus, excluding the nucleoli. The SSX2 protein exhibited a diffuse staining pattern whereas both the SYT and SYT–SSX proteins appeared in several nuclear dots. Similar nuclear dots were also detected in primary synovial sarcoma cells growing in a short-term *in vitro* culture. Double immunofluorescence in conjunction with confocal laser-scanning microscopy revealed that the SYT and SYT–SSX nuclear dots do not co-localize with known nuclear structures as e.g. coiled bodies, SC35 interchromatin granules or PML bodies. The similar nuclear localization patterns of SYT and SYT–SSX suggest that the SYT–SSX fusion proteins are directed to SYT-associated nuclear domains where an abnormal function may be exerted.

INTRODUCTION

Cytogenetic abnormalities, numerical and structural, are frequently found in human hematopoietic and solid tumors. Mesenchymal tumors often carry chromosomal translocations, sometimes as a single cytogenetic anomaly (1). The recent molecular cloning of the corresponding translocation breakpoints has revealed that segments of two distinct genes become juxtaposed, resulting in the generation of chimeric proteins with new functional properties (2,3). Generally, the DNA binding domain of a transcription factor becomes fused to a transactivating domain of another transcription factor, resulting in a chimeric protein with altered transcription regulating activity.

The functional consequences associated with the production of chimeric proteins have been studied most extensively in peripheral primitive neuroectodermal tumors of childhood, also designated as Ewing tumors. These malignancies are characterized by the presence of variant translocations with consistent involvement of the EWS gene, from the chromosomal region 22q12, and members of the ETS family of transcription factors, such as FLI1, ERG, ATV1, E1AF or FEV (3–5). The precise function of the EWS protein has not yet been elucidated although it has been found that a carboxyl-terminal domain is able to bind RNA and an amino-terminal domain exhibits transactivating properties (6). As a consequence of the chromosomal translocations, the carboxyl-terminal RNA binding domain of EWS is replaced by the DNA binding domain of a transcription factor. This results in a hybrid protein with altered transcriptional activation properties and a strong transforming potential, as has been demonstrated for the EWS–FLI1 fusion protein (6–8).

Also in synovial sarcomas, a recurrent chromosomal translocation, t(X;18)(p11.2;q11.2), has been found (9). The t(X;18)(p11.2;q11.2), or complex variants thereof, frequently occurs as the sole cytogenetic abnormality and is presumably causally related to synovial sarcoma development (1,9,10). Recently, using somatic cell hybrids and fluorescence *in situ* hybridization analysis with yeast artificial chromosome and cosmid probes, we and others have identified two alternative and mutually exclusive Xp11.2 breakpoints, suggesting that a single gene on chromosome 18 may be fused to one of two different genes on the X chromosome (11–15). This hypothesis was subsequently validated by the cloning of the t(X;18) breakpoints, showing that a novel gene, SYT on chromosome 18, is disrupted and juxtaposed to one of two related but distinct novel genes, SSX1 or SSX2 on the X chromosome (16–19).

At present, the biological function of the SYT protein is still elusive. The SYT amino acid sequence is composed of 387 residues, being rich in glutamine, proline and glycine (19, 16 and 14%, respectively), and comparison with known protein sequences from databases provided scant information about possible functional domains. Only three potential SH2 binding domains, one potential SH3 binding domain and an annexin-like imperfect direct repeat could be recognized in the SYT sequence (17,20). In addition, SYT contains several stretches of glutamine and proline residues which may be indicative of a role in transcription

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activation (21). Evaluation of mRNA levels in human and mouse tissues has shown that the SYT gene is widely expressed (17,20). mRNA *in situ* hybridization studies uncovered that in early mouse development, SYT is predominantly expressed in certain cartilaginous and neuronal tissues and that in adult mice, testicular SYT expression is restricted to primary spermatocytes (20).

Several distinct but highly homologous SSX genes have been identified on the X chromosome (22). Full-length cDNAs of three members of this gene family have thus far been cloned, and all were found to encode putative proteins of 188 amino acids (19,22). Inspection of the amino acid sequences of the SSX proteins disclosed an acidic carboxyl-terminal domain, an amino-terminal region with homology to a Krüppel-associated box (KRAB) domain, and consensual sequences for N-glycosylation and tyrosine phosphorylation sites (19,22). The KRAB domain has been identified in a subgroup of zinc finger proteins, where it functions as a transcriptional repressor (23,24). Even though SSX proteins do not contain DNA binding sequences, the presence of an acidic domain and a putative KRAB domain indicates that they may be involved in transcription regulation. In contrast to the ubiquitous SYT expression, SSX transcripts have only been detected in the testis and, at lower levels, the thyroid gland (17,19). Therefore, these expression studies indicate that at least one consequence of the SYT–SSX fusion is the ectopic expression of SSX sequences in synovial sarcoma precursor cells. Moreover, it was recently shown that ectopic SSX2 expression may occur in several different types of tumors, suggesting that additional mechanisms other than fusion with SYT may be at work (25).

In the majority of synovial sarcoma cases, SYT–SSX fusion genes encode proteins comprised of the first 379 amino acids of SYT juxtaposed to the last 78 amino acids of either SSX1 or SSX2, thereby excluding the putative KRAB domain (18,19,26). Less frequent variant fusion genes have also been identified (18,19,26). Since most of the SYT sequence is usually present in the hybrid proteins, it is conceivable that addition of the carboxyl-terminal segment of SSX will impart a new or modified function to the SYT protein, thereby triggering tumor development.

Here, we report the production of specific polyclonal antibodies against the SYT and SSX proteins. These antibodies were used to determine the subcellular localization of these proteins and their respective fusion products in transfected COS-1 cells and primary tumor cells.

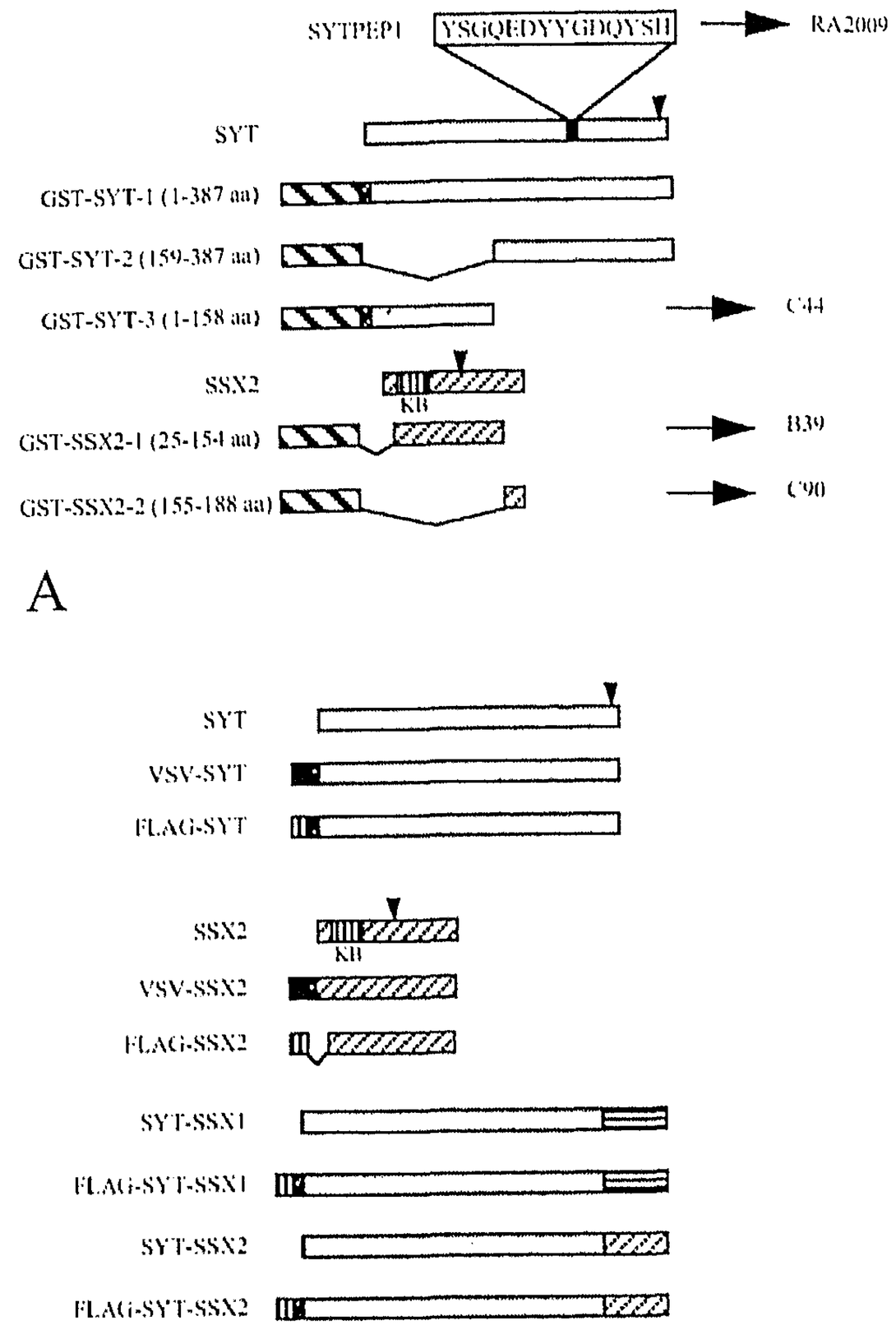

Figure 1. Schematic representation of the SYT, SSX2, and SYT–SSX constructs. (A) GST fusion constructs containing total SYT (GST-SYT-1), C-terminal SYT (GST-SYT-2), N-terminal SYT (GST-SYT-3), a central segment of SSX2 (GST-SSX2-1) or C-terminal SSX2 (GST-SSX2-2). The GST–SYT-3, GST–SSX2-1, and GST–SSX2-2 proteins were used to generate antibodies C44, B39, and C90, respectively. A SYT 14-amino acid peptide (SYTPEP1) was used to generate rabbit polyclonal antibody RA2009. (B) SYT, SSX2, SYT–SSX1, and SYT–SSX2 cDNAs cloned into appropriate eukaryotic expression vectors enabling the production of tagged (VSV, FLAG) and untagged proteins. Arrowheads indicate the most common breakpoints, resulting in the SYT–SSX fusion proteins, as depicted.

RESULTS

Production and specificity of antibodies against SYT and SSX2

As a first approach towards the elucidation of the function of the synovial sarcoma t(X;18)-associated gene products, we generated polyclonal antibodies against the SYT and SSX proteins. Full-length SYT and SSX2 cDNAs were isolated and different segments of these cDNAs were cloned into pGEX vectors to produce glutathione-S-transferase (GST) fusion proteins (Fig. 1A). Since the SSX1 and SSX2 proteins are highly homologous (81% identity), we only used one of them, SSX2, to generate an anti-SSX polyclonal antiserum. A GST–SYT fusion protein containing the first 158 amino acids of SYT (designated GST–SYT-3) (Fig. 1A) was purified from *Escherichia coli* and used to generate rabbit polyclonal antiserum C44. Similarly, two GST–SSX2 fusion proteins containing amino acids 25–154 (GST–SSX2-1) and amino acids 155–188 (GST–SSX2-2) were expressed and purified from *E.coli* and used to generate rabbit antisera B39 and C90, respectively (Fig. 1A). Additionally, a polyclonal antiserum (RA2009) was raised against a synthetic 14 amino acid peptide from the SYT carboxyl-terminal region. All polyclonal antibodies were tested by immunoblotting and shown to specifically recognize the antigens against which they were raised (Fig. 2A and data not shown).

The deduced amino acid sequences of the SYT, SSX, and SYT–SSX proteins predict molecular weights of 49, 25 and 58 kDa, respectively. However, *in vitro* transcription and translation of the respective cDNAs yielded proteins with slower mobilities after SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis:

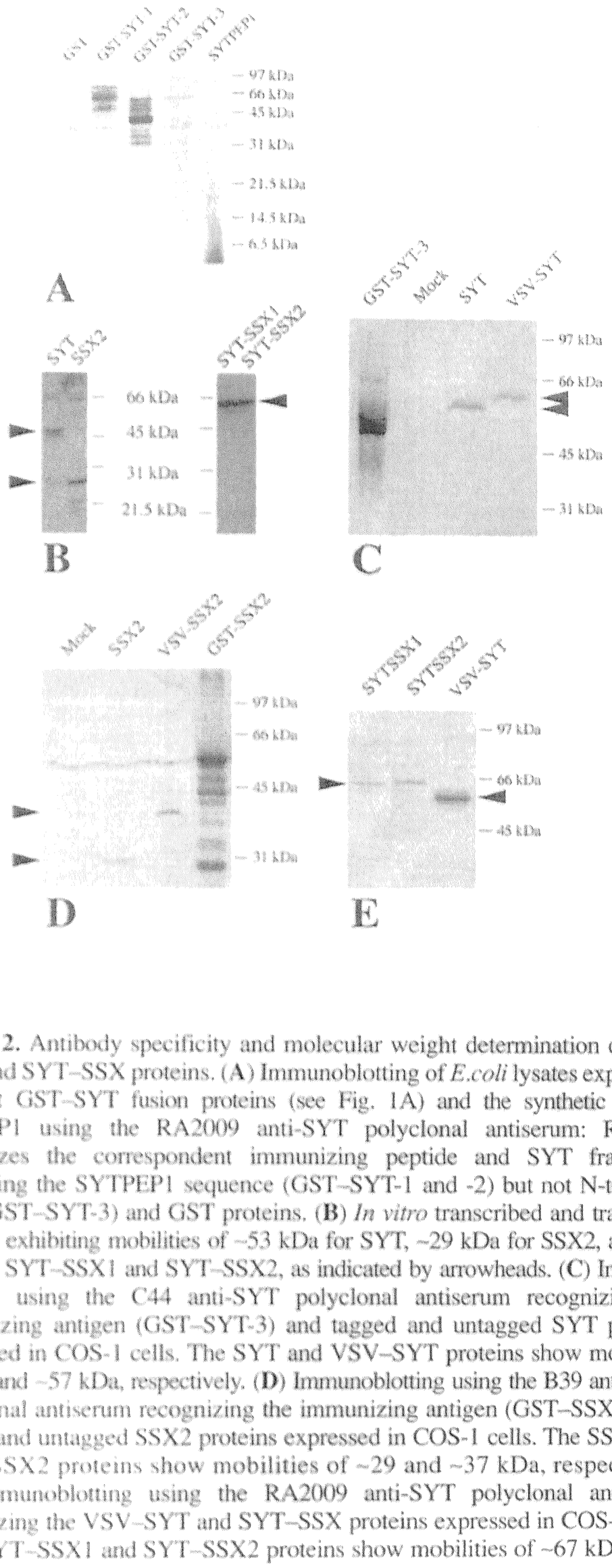

Figure 2. Antibody specificity and molecular weight determination of SYT, SSX, and SYT–SSX proteins. (**A**) Immunoblotting of *E.coli* lysates expressing different GST–SYT fusion proteins (see Fig. 1A) and the synthetic peptide SYTPEP1 using the RA2009 anti-SYT polyclonal antiserum: RA2009 recognizes the correspondent immunizing peptide and SYT fragments containing the SYTPEP1 sequence (GST–SYT-1 and -2) but not N-terminal SYT (GST–SYT-3) and GST proteins. (**B**) *In vitro* transcribed and translated proteins exhibiting mobilities of ~53 kDa for SYT, ~29 kDa for SSX2, and ~67 kDa for SYT–SSX1 and SYT–SSX2, as indicated by arrowheads. (**C**) Immunoblotting using the C44 anti-SYT polyclonal antiserum recognizing the immunizing antigen (GST–SYT-3) and tagged and untagged SYT proteins expressed in COS-1 cells. The SYT and VSV–SYT proteins show mobilities of ~53 and ~57 kDa, respectively. (**D**) Immunoblotting using the B39 anti-SSX2 polyclonal antiserum recognizing the immunizing antigen (GST–SSX-2) and tagged and untagged SSX2 proteins expressed in COS-1 cells. The SSX2 and VSV–SSX2 proteins show mobilities of ~29 and ~37 kDa, respectively. (**E**) Immunoblotting using the RA2009 anti-SYT polyclonal antiserum recognizing the VSV–SYT and SYT–SSX proteins expressed in COS-1 cells. Both SYT–SSX1 and SYT–SSX2 proteins show mobilities of ~67 kDa.

~53 kDa for SYT, ~29 kDa for SSX2 and ~67 kDa for SYT–SSX (Fig. 2B). The SYT–SSX1 and SYT–SSX2 proteins showed indistinguishable electrophoretic mobilities, which is in keeping with their identical amino acid numbers.

In order to detect the respective proteins *in vivo*, COS-1 cells were transiently transfected with SYT, SSX2, SYT–SSX1, and SYT–SSX2 cDNAs. The different anti-SYT and anti-SSX2 antibodies were then used to detect the exogenous proteins in cell extracts by immunoblotting. Both the C44 and RA2009 anti-SYT antibodies recognized a ~53 kDa protein in SYT-transfected but not in untransfected COS-1 cells (Fig. 2C and data not shown). Similarly, the B39 anti-SSX antibody detected a ~29 kDa SSX2 protein in transfected but not in untransfected COS-1 cells (Fig. 2D).

The anti-SYT antibodies were also able to detect exogenously expressed ~67 kDa SYT–SSX1 and SYT–SSX2 proteins (Fig. 2E). Since the mobilities of the *in vivo* expressed proteins were not significantly different from the *in vitro* produced counterparts, we infer that no major post-translational modifications occur in COS-1 cells.

Fusion proteins carrying amino-terminal peptide tags, either the vesicular stomatitis viral (VSV) tag or the FLAG tag (Fig. 1B), were also expressed in transiently transfected COS-1 cells. Both anti-tag monoclonal antibodies and anti-SYT or anti-SSX polyclonal antibodies revealed equivalent bands, with slower mobilities as compared to the corresponding untagged proteins, as expected (Fig. 2C,D). These results confirm that the polyclonal antibodies detect proteins derived from the transfected cDNAs and not any other spurious proteins. The rabbit pre-immune sera did not detect any SYT or SSX-like proteins (data not shown).

Nuclear localization pattern of SYT and SSX2 proteins

We performed indirect immunofluorescence assays to detect untagged or VSV- or FLAG-tagged SYT and SSX2 proteins in transfected COS-1 cells using anti-target and anti-tag antibodies.

Expressing either tagged or untagged SSX2, transfected but not untransfected cells showed a consistent diffuse staining pattern throughout the nucleus, with exclusion of the nucleoli (Fig. 3A–C). To evaluate the specificity of the B39 antibody staining, we carried out competition experiments with specific and unspecific antigens. Pre-incubation of B39 antiserum with the immunizing antigen, GST–SSX2-1, resulted in the absence of nuclear staining in FLAG–SSX2 expressing COS-1 cells, as verified by co-staining with anti-FLAG antibody (Fig. 3D–F). In contrast, pre-incubation with an unspecific protein did not alter the B39 staining pattern (Fig. 3G–I).

Similarly, the antibodies raised against SYT, either C44 or RA2009, were used for indirect immunofluorescence analysis of COS-1 cells expressing tagged or untagged SYT proteins. Independent of the presence of a peptide tag, repeated experiments disclosed a consistent nuclear labeling of transfected but not untransfected COS-1 cells (Fig. 3J–L). SYT labeling revealed a prominent punctated pattern standing out from a diffuse staining throughout the nucleoplasm but excluding nucleoli (Fig. 3J–L). In addition, cell-to-cell variability was observed regarding size, intensity and number of nuclear dots, ranging from 20 to 50. Moreover, we could observe that SYT dots were not restricted to a particular nuclear region, even though dots bordering the nucleoli were often detected (Fig. 3K). These results were obtained after a short post-transfection period of incubation (16–18 h) since longer periods of time (40–48 h) resulted in the accumulation of high amounts of SYT protein in lipoprotein inclusions, as observed by electron microscopy (data not shown). Again, pre-incubation of both the RA2009 and the C44 antibodies with their respective immunizing antigens resulted in the abrogation of the specific nuclear staining in immunofluorescence assays. In contrast, incubation with unspecific proteins did not alter the SYT staining pattern (data not shown).

To confirm that our results were not influenced by the formaldehyde/methanol fixation method used (see Materials and Methods), cells were fixed with either methanol alone, formaldehyde followed by Triton X-100, or acetone. In all cases, similar localization patterns were observed (data not shown).

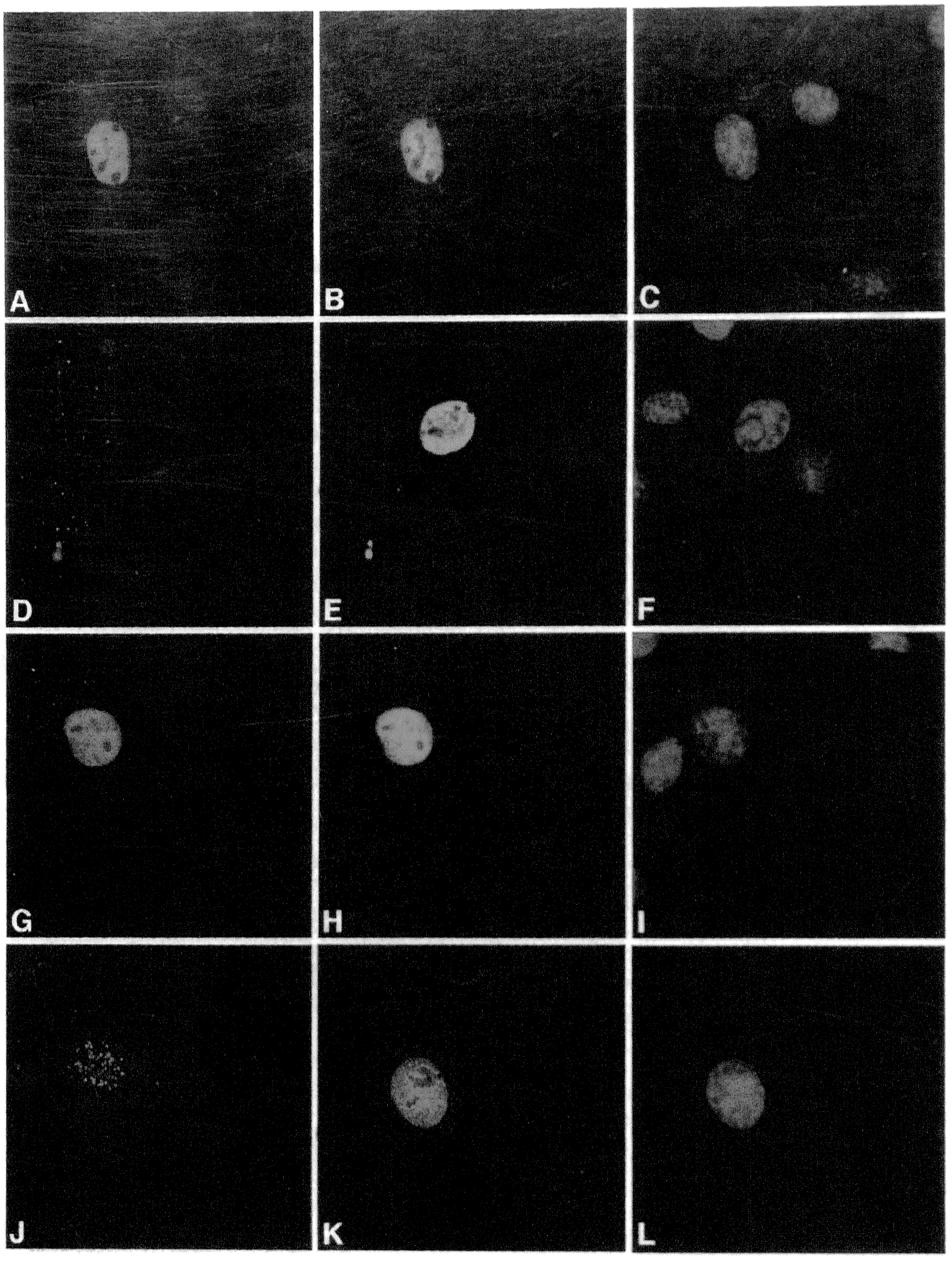

Figure 3. Indirect immunofluorescence analysis of COS-1 cells transfected with SSX2 and SYT cDNA constructs. Panels A to I show COS-1 cells transfected with FLAG–SSX2. Transfected cells were incubated with anti-FLAG M5 mAb (**B**, **E**, and **H**) and, simultaneously, with either B39 antibody (**A**), B39 pre-incubated with GST–SSX2 (**D**), or B39 pre-incubated with GST–SYT-3 (**G**). (**C**, **F**, and **I**) DAPI counterstaining. Panels J to L show VSV–SYT transfected COS-1 cells incubated with the anti-VSV P5D4 mAb (**J** and **K**). (**L**) DAPI counterstaining of the cell shown in (K). Original magnification: 630×.

SYT–SSX chimeric proteins exhibit nuclear localization patterns similar to SYT

SYT–SSX1 or SYT–SSX2 constructs (Fig. 1B) were transfected into COS-1 cells and immunofluorescence detection with anti-SYT antibodies disclosed that both fusion proteins were present in the nucleus, displaying a punctated pattern closely resembling that of SYT (Fig. 4A–D). The similarities between the SYT–SSX and SYT results included the cell-to-cell variability in number and size of the nuclear dots, the presence of a diffuse nucleoplasmic staining accompanying the punctated pattern and the localization of several dots adjacent to the nucleoli. Again, FLAG-tagged SYT–SSX proteins revealed similar staining patterns and competing out the antibodies with immunizing antigens resulted in the expected loss of specific signals (data not shown). Immunodetection of SYT–SSX2 proteins with both anti-SSX2 antibodies also revealed punctated nuclear patterns, indicating that the observed signals reflect genuine SYT–SSX expression and not that of SYT alone (Fig. 4E, F and data not shown).

Immunofluorescence analysis using the anti-SYT RA2009 antibody was also performed on primary tumor cells derived from

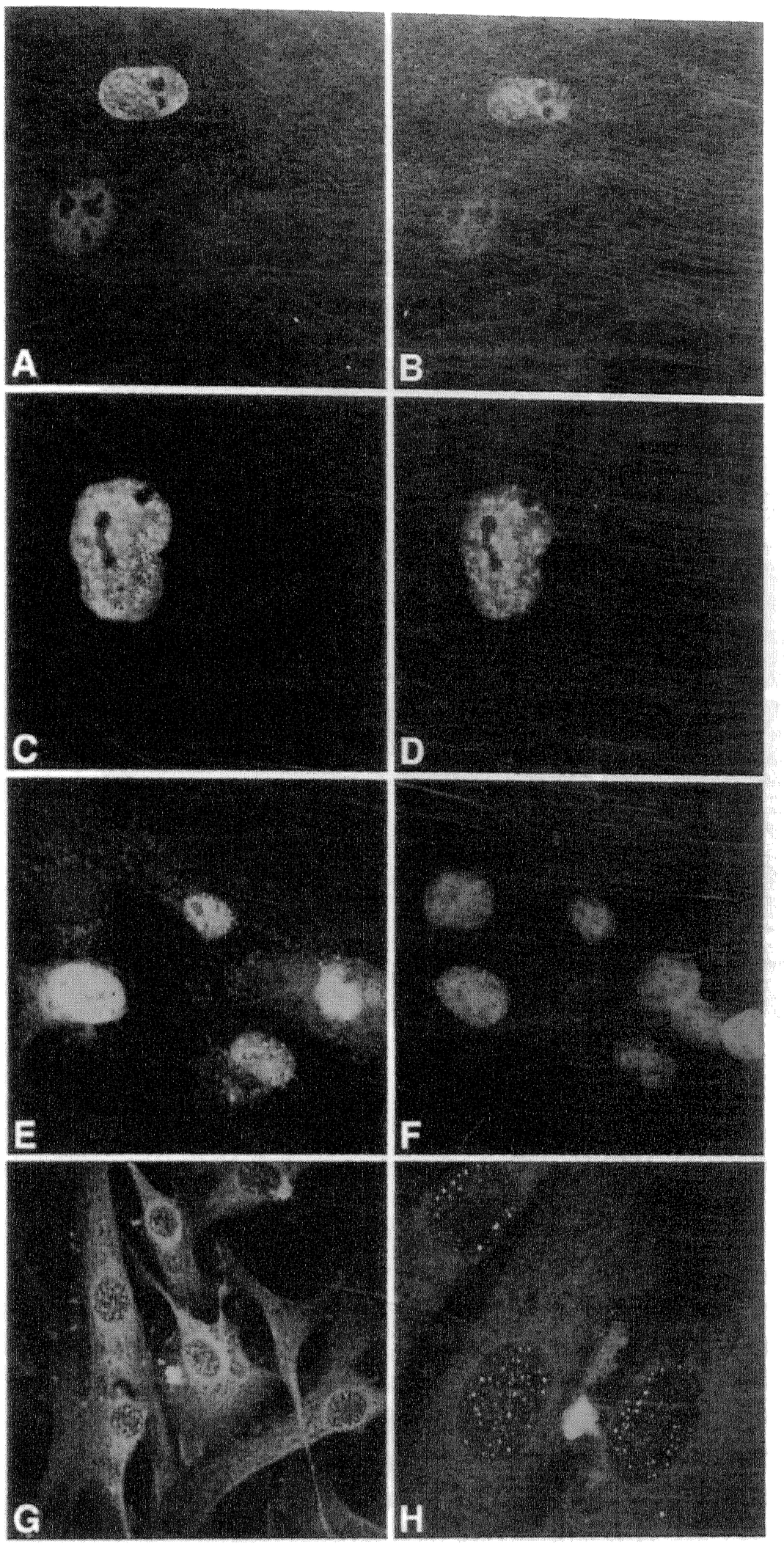

Figure 4. Subcellular localization of SYT–SSX proteins in transfected COS-1 cells and in synovial sarcoma cells. FLAG–SYT–SSX2 protein expressed in COS-1 cells is recognized both by the anti-SYT C44 antibody (anti-SYT) (**A**) and anti-FLAG M5 antibody (**B**). FLAG–SYT–SSX1 protein overexpressed in COS-1 cells is recognized both by anti-SYT C44 antibody (**C**) and anti-FLAG M5 antibody (**D**). The anti-SSX2 C90 antibody also stains cells overexpressing SYT–SSX2 protein but not untransfected cells (**E**, **F**). (**G**, **H**) Synovial sarcoma cells grown *in vitro* and immunolabeled with the anti-SYT RA2009 antibody. A nuclear punctated pattern was visible in all cells studied. Original magnifications: (A, B, E, F) 630×; (C, D) 1000×; (G) 400×; (H) 1000×.

a biphasic synovial sarcoma, carrying a SYT–SSX1 fusion gene, which was grown in a short-term *in vitro* culture. The tumor cells exhibited a nuclear punctated staining pattern, displaying 40–70 dots distributed throughout the nucleoplasm excluding nucleoli (Fig. 4G, H), hence resembling the SYT/SYT–SSX labeling pattern observed in COS-1 cells. The size of the labeled foci was smaller in the primary tumor cells in comparison with those observed in the COS-1 cells, possibly as a result of a lower level of expression. Despite this, the detection of a nuclear punctated staining pattern in primary synovial sarcoma cells supports the results obtained with the exogenously expressed proteins in transiently transfected COS-1 cells.

SYT and SYT–SSX dots do not co-localize with known nuclear bodies

Our findings show that both the SYT and SYT–SSX proteins are present in dots distributed throughout the nucleoplasm, with exception of the nucleoli. At present, different nuclear bodies in the interchromatin compartment have been identified on the basis of their morphological appearance and composition. Four main types of nuclear bodies have been characterized by detection of its components with specific antibodies: coiled bodies, containing p80 coilin, fibrillarin and splicing factors such as the U1, U2, U4/U6 and U5 snRNPs; PML bodies, including the PML and Sp100 proteins; GATA transcription factor-containing nuclear bodies; and RNA cleavage bodies (27). In the interphase nucleus, the snRNPs are not only present in coiled bodies (1–5 per cell) but also in clusters of interchromatin granules (20–50 per cell) and perichromatin fibrils which are revealed by immunolabeling as foci, speckles and diffuse staining, respectively (28).

To evaluate whether the SYT and SYT–SSX dots are associated with known nuclear domains, we performed double immunofluorescence assays using mouse monoclonal antibodies raised against U-snRNPs (mAbs 4G3, and 9A9), Sm antigens (mAb Y12), SC35 splicing factor (mAb anti-SC35), and PML protein (mAb 5E10). No significant co-localization was detected between the SYT/SYT–SSX domains and any of the nuclear bodies detected by the different antibodies in COS-1 cells (Fig. 5). Some degree of co-localization could be observed, for instance, with anti-SC35 (Fig. 5E, yellow signals), but this was not a consistent feature in the cells examined.

DISCUSSION

Polyclonal antibodies against the SYT and SSX2 proteins were generated and were shown to specifically detect exogenously expressed SYT, SSX2, and synovial sarcoma-associated SYT–SSX proteins in COS-1 cells, either by immunoblotting or by immunofluorescence assays. The examined proteins exhibited electrophoretic mobilities in conformity with the predicted molecular weights: ~53 kDa for SYT, ~29 kDa for SSX2 and ~67 kDa for SYT–SSX1 and SYT–SSX2, and were found to be located in the nucleus. The addition of peptide tags (FLAG or VSV) to the amino-termini of these proteins allowed the simultaneous use of polyclonal antibodies against the target proteins and monoclonal antibodies against the tag peptides. In all cases, both types of antibodies resulted in similar staining patterns after double immunofluorescence analyses, thus confirming that the nuclear labeling was derived from the exogenous proteins. These results also show that the use of an amino-terminal tag, either FLAG or VSV, does not interfere with the subcellular localization of the target proteins. Furthermore, antibody competition experiments were performed and, invariably, pre-incubation with immunizing antigens resulted in loss of specific nuclear labeling whereas unrelated antigens had no effect on the staining pattern.

The localization studies reported here were performed in COS-1 cells. Similar experiments carried out with HeLa cells revealed essentially the same results (data not shown). In both

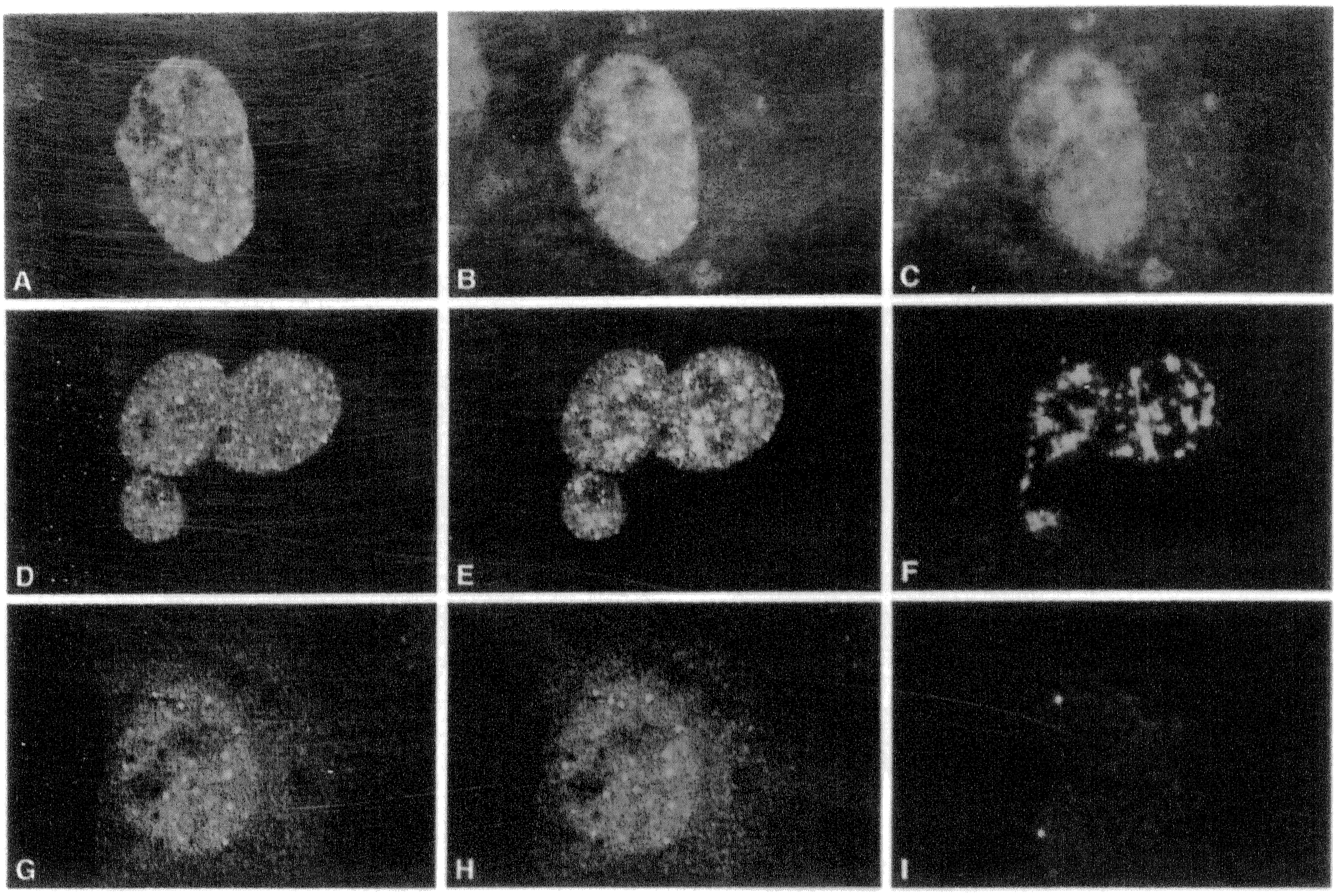

Figure 5. Double labeling of SYT or SYT–SSX dots and other nuclear domains in COS-1 cells visualized by confocal laser-scanning microscopy. (**A–C**) FLAG–SYT–SSX1 transfected cell stained with C44 (anti-SYT) (A) and Y12 (anti-Sm) (C) antibodies. (**D–F**) SYT–SSX2 transfected cell stained with RA2009 (anti-SYT) (D) and anti-SC35 (F) antibodies. (**G–I**) SYT transfected cell stained with RA2009 (G) and 5E10 (anti-PML) (I) antibodies. (B, E, H) Depiction of overlaid images. Original magnifications: 2400×.

cases, the SSX2 protein appeared diffusely distributed throughout the nucleoplasm whereas the SYT protein exhibited a punctated or speckled pattern against a somewhat diffuse staining, excluding the nucleoli. Since the SYT protein has a high content of glutamines and prolines, it seems plausible that it may function as a transcriptional activator (21). Also, the SSX proteins may play a role in the regulation of gene expression since they contain a putative Krüppel-associated box, a domain which has been shown to repress transcription when tethered to the DNA (23,24). However, SSX proteins seem to lack a DNA binding domain, so a role in gene expression may be executed via interaction with other proteins, as has also been suggested for the t(12;16)-associated CHOP oncoprotein (29). Taken together, these findings lend further support for a putative role of these proteins in transcription regulation.

In addition, we have found that the SYT–SSX chimeric proteins display a nuclear distribution pattern that parallels the one observed for SYT. The similarity between the SYT and SYT–SSX localization patterns comprehended the cell-to-cell variability in the number and size of nuclear dots and the frequent perinucleolar localization. Based on these findings, we assume that in synovial sarcoma cells the SYT–SSX proteins are targeted to the nuclear domains where SYT normally resides, in this way interfering with or modifying the normal function of SYT. Further studies have to be carried out to elucidate whether these SYT/SYT–SSX nuclear domains are involved in gene regulation and/or cellular growth/differentiation regulating processes.

So far, no nuclear localization signal (NLS) sequences have been recognized in SYT and SSX (17,19). However, the observed nuclear localization of these proteins prompted us to search for sequences which could account for their targeting to the nucleus. NLSs are characterized by a high content in basic amino acids (lysine or arginine) and may be subdivided in single and bipartite NLSs (30). Bipartite NLSs include two clusters of basic amino acids, one with two basic residues and another one with at least three basic residues in a stretch of five, interposed by a spacer of ~10 random amino acids (30). The SYT amino acid sequence is poor in lysines and arginines and a closer inspection only uncovered a putative five-amino acid long non-typical NLS (31) at its amino-terminal end, starting at amino acid 9: RQRGK. In contrast to SYT, the SSX proteins exhibit a high number of lysine and arginine residues, enabling us to pinpoint three putative bipartite NLSs distributed along the protein sequence, that are conserved between the different SSX proteins (22) (Fig. 6). Since the FLAG–SSX2 construct that we used does not contain NLS1 but nevertheless resulted in nuclear staining, the latter may be excluded at least as an essential NLS. NLS3 fulfils the above-mentioned bipartite NLS criteria and is highly conserved between the different SSX family members (Fig. 6), so this sequence may be the best candidate to act as an authentic NLS. Mutagenesis studies are required to establish which of the putative peptide sequences in SYT and SSX are involved in nuclear targeting.

NLS1 (aa 9-23)

SSX1	...KRPRDDAKASEKRSK...
SSX2	...RRPT VGAQ I PEKIQK...
SSX3	...RRPT VGAQ I PEKIQK...

NLS2 (aa 52-64)

SSX1	...KRNYKAMTKLGFK...
SSX2	...KRKYEAMTKLGFK...
SSX3	...KRKYEAMTKLGFK...

NLS3 (aa 158-172)

SSX1	...KRGKHAWTHRLRERK...
SSX2	...KRGEHAWTHRLRERK...
SSX3	...KRGEHAWTHRLRERK...

Figure 6. Putative bipartite nuclear localization signals found after inspection of the SSX1, SSX2, and SSX3 protein sequences (22). Basic amino acids (lysine and arginine) are shown in bold type and the two basic amino acid clusters identified according to Dingwall and Laskey (30) are underlined.

The finding that both SYT and SYT–SSX proteins are localized in particular subnuclear domains is of interest since other cancer-associated proteins, such as PML, WT1, BRCA1, Rb, and TP53, have also been found to be present in subnuclear domains (32–37). For instance, the BRCA1 protein displays a distinct subnuclear distribution pattern related to the cell cycle phase and has been associated with meiotic synaptonemal complexes (37). Furthermore, the Rb protein is bound to the nuclear matrix during early G1 phase, being particularly concentrated at the nuclear periphery and in the nucleolus (34). The PML protein is normally localized in nuclear bodies, in association with other proteins (38). However, when expressing the PML–RARα fusion protein, acute promyelocytic leukemia cells present morphologically altered PML bodies (33,35). The PML-associated nuclear bodies represent one subtype among several nuclear bodies which have been characterized morphologically and biochemically and include coiled bodies, GATA nuclear bodies and RNA cleavage bodies (27). The coiled bodies were the first to be characterized and are composed of factors such as p80 coilin, U-snRNPs (U1, U2, U4/U6, and U5), and Sm-proteins (27,28). In this report, double immunofluorescence assays indicated that the SYT and SYT–SSX domains do not co-localize with either coiled bodies (mAbs 4G3, 9A9, and Y12), splicing factor interchromatin granules (mAbs 4G3, 9A9, Y12, and anti-SC35) or PML bodies (mAb 5E10). This finding suggests that the SYT/SYT–SSX dots are associated with a new class of subnuclear domains. Further studies will have to be performed to disclose what the function of such nuclear domains may be in normal and abnormal (cancer) cellular growth.

MATERIALS AND METHODS

Isolation of cDNAs and plasmid constructs

The human fibrosarcoma HT-1080 and the testis 5′ stretch cDNA libraries from Clontech were employed for screening, using essentially the same procedures as described before (12,22). Several clones were isolated and full-length SYT and SSX2 cDNAs were subcloned into pT7T3 (Pharmacia) and pGEM7zf (Promega) cloning vectors for further manipulations. The SYT–SSX1 and SYT–SSX2 cDNAs were obtained by ligating restriction digested SYT–SSX RT-PCR products into a SYT cDNA cloned in the pT7T3 vector.

For bacterial expression of glutathione-S-transferase (GST) fusion proteins, cDNA fragments from the SYT and SSX2 genes were cloned in-frame into pGEX vectors (Amrad). A full-length SYT cDNA, a carboxyl-terminal fragment including amino acids 159 to 387, and a fragment including the first 158 amino acids were cloned in-frame into an appropriate pGEX vector (see Fig. 1A). SSX2 fragments including amino acids 25–154 and amino acids 155–188 were also cloned into appropriate pGEX vectors (Fig. 1A).

For eukaryotic expression, the full-length SYT cDNA was cloned into the *Eco*RI site of the pSG5 vector (Stratagene). The SYT cDNA was tagged with the vesicular stomatitis virus (VSV) glycoprotein sequence (YTDIEMNRLGK) by in-frame cloning between the *Sma*I and *Xho*I sites of the pSG8-VSV vector (derived from the pSG5 vector and kindly provided by Dr Edwin Cuppen). In addition, The SYT cDNA was tagged with the FLAG tag (DYKDDDDK) by inserting in-frame the same *Sma*I–*Xho*I SYT fragment into the pSuperCATCH vector (kindly provided by Dr C. Hovens). This vector is derived from the pCATCH vector but bears an extended multiple cloning site (39). The full SSX2 cDNA was cloned between the *Eco*RI and *Hin*dIII sites of the pSG8 vector and into the *Sma*I site of the pSG8–VSV vector. Furthermore, a *Stu*I–*Sal*I SSX2 fragment was cloned between the *Eco*RV and *Sal*I sites of the pCATCH vector, so deleting the first 24 amino acids of the SSX2 cDNA. The SYT–SSX1 and SYT–SSX2 chimeric cDNAs were inserted between the *Eco*RI and *Hin*dIII sites of the pSG8 vector. These cDNAs were also FLAG tagged by in-frame insertion of the SYT–SSX1 cDNA between the *Eco*RI and *Xba*I sites of the pSuperCATCH/BF vector, a pSuperCATCH vector with a filled-in *Bam*HI site, and the SYT–SSX2 cDNA between the *Sma*I and *Sph*I sites of the pSuperCATCH vector. The correct orientation and in-frame cloning of the constructs was confirmed by restriction digestion analysis and DNA sequencing. The corresponding proteins are schematically depicted in Figure 1. Sequencing was performed in both orientations using vector-specific oligonucleotides and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) and an automated DNA sequencer (ABI 373A, Applied Biosystems).

Bacterial expression and generation of polyclonal antibodies

The GST–SSX2 fusion proteins were expressed and purified following published methods (40). Briefly, logarithmically grown *E.coli* DH5α cells carrying the pGEX–SSX2 constructs were induced with 0.1 mM of isopropyl-β-D-thiogalactopyranoside for 3–5 h at 37°C. The cells were pelleted, resuspended in 1/25 volume of ice-cold PBS, and lysed by sonication. Triton X-100 (Fluka) was added to a final concentration of 1%, and then the lysate was centrifuged at 5000 *g* to pellet insoluble material.

Glutathione-agarose beads (Sigma) were added to the supernatant fraction, incubated for 30 min at 4°C, and washed three times with PBS. To elute GST–SSX2 fusion proteins, the beads were resuspended in 5 mM reduced glutathione (Sigma)/50 mM Tris pH 8.0 solution and incubated with agitation for at least 2 min at room temperature.

Probably due to the hydrophobic nature of the SYT protein, the GST–SYT fusion proteins were not amenable to solubilization by the previous method (data not shown). Thus, instead we used another method adapted from Frangioni and Neel (41) through which GST–SYT-3, but not GST–SYT-1 and -2, were purified. Briefly, expression was carried out as mentioned above, the bacterial pellet was washed with ice-cold STE buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA) and resuspended in 1/50 the volume of the bacterial culture in ice-cold STE buffer containing 100 µg/ml of lysozyme. After incubating for 15 min on ice, dithiothreitol, phenylmethylsulphonyl fluoride (PMSF), and *N*-lauroylsarcosine were added to final concentrations of 5 mM, 1 mM, and 1.5%, respectively, and the mixed suspension was lysed by sonication. The lysate was centrifuged at 11 000 *g* to remove insoluble debris, and Triton X-100 was added to the supernatant to a final concentration of 4%. Glutathione-agarose beads were added to the lysate, and the GST–SYT-3 protein was purified as mentioned above. The concentration and purity of the eluted proteins were estimated by conventional SDS–PAGE. The GST fusion proteins were then applied for rabbit immunization following standard procedures, resulting in the antisera C44 (anti-GST–SYT-3), B39 (anti-GST–SSX2-1), and C90 (anti-GST–SSX2-2). The antiserum RA2009 was obtained by immunizing rabbits with a synthetic 14 amino acid SYT peptide corresponding to residues 264–277 (YSGQEDYYGDQYSH) (Genosys Biotechnologies Inc.). All antisera were tested on Western blots to evaluate their specificity and were affinity purified using Affi-Gel Protein A agarose (Bio-Rad), according to the manufacturer's recommendations, to be used in immunofluorescence assays.

Western blot analysis

Transfected COS-1 cells were lysed following standard methods (42). Briefly 40–48 h after transfection, cells were washed twice with ice-cold PBS and lysed by incubating for 30 min in ice-cold RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.5% Na deoxycholate, 0.1% SDS, 0.1% 2-mercaptoethanol, 1 mM PMSF, 4.8 µg/ml aprotinin). The cells were then scraped off and spun down at 12 000 *g* for 2 min at 4°C. Supernatants were collected and aliquots were mixed with a 5× SDS–PAGE sample buffer (62.5 mM Tris pH 6.8, 20% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.025% bromophenol blue), heat denatured, and analyzed on 10 or 12% SDS–PAGE gels. Electrophoresed samples and unstained molecular markers (Bio-Rad) were then electroblotted onto nitrocellulose filters (Schleicher & Schuell) in transfer buffer (192 mM glycine, 25 mM Tris, 20% ethanol), using a Bio-Rad Mini Trans-Blot Cell. The filters were stained with 0.2% Ponceau S (Sigma) to assess the efficiency of blotting and afterwards blocked (0.4% gelatin, 10 mM Tris pH 7.5, 350 mM NaCl) for 30 min at 37°C. After blocking, filters were incubated with the primary antibody in RIA buffer (10 mM Tris pH 7.5, 160 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate) for 1 h at room temperature. As primary antibodies, we used the anti-SYT (C44, 1:1000 dilution and RA2009, 1:1000 dilution) and anti-SSX2 (B39, 1:5000 dilution and C90, 1:3000 dilution) polyclonal antibodies and the anti-VSV [P5D4, 1:1500 diluted (43)] and anti-FLAG (M5, 1:300 diluted, Eastman Kodak Company) monoclonal antibodies. The filters were washed three times in RIA buffer and incubated with alkaline phosphatase-conjugated secondary antibodies (1:3000 dilution, Bio-Rad) in RIA buffer, for 1 h at room temperature. The filters were developed using the Alkaline Phosphatase Conjugate Substrate Kit according to manufacturer's instructions (Bio-Rad).

In vitro transcription and translation

In vitro transcription and translation reactions were performed with the TnT Coupled Reticulocyte Lysate Systems and tRNA^{nscend} Non-Radioactive Translation Detection System from Promega, following the manufacturer's protocols.

Synovial sarcoma cells

Tumor fragments obtained from a biphasic synovial sarcoma resected from a 43-year-old male were transplanted s.c. into the inter-scapular region of nude mice (N:NIH[s] – nu/nu) maintained under specific-pathogen-free conditions. When the tumor volume reached ~1500 mm^3, it was resected and divided into small fragments 5–6 mm diameter and reimplanted s.c. into nude mice or transferred to culture plates in RPMI medium, supplemented with 16% fetal calf serum and antibiotics.

Transfections and indirect immunofluorescence assays

Green monkey kidney COS-1 cells and human cervix carcinoma HeLa cells were grown in DMEM containing 10% fetal calf serum and were transiently transfected by electroporation in 0.4 cm cuvettes (Eurogentec) (125 µF, 300 V), using a Bio-Rad Gene Pulser apparatus. Transfected COS-1 or HeLa cells or synovial sarcoma cells were grown on poly-L-lysine (Sigma) coated coverslips for 40–48 h (16–18 h for SYT-transfected cells), fixed with 3% formaldehyde in PBS for 30 min at room temperature, washed three times with PBST solution (PBS, 0.05% Tween 20), permeabilized with methanol at –20°C for 10 min, and washed once with PBST. The coverslips were then incubated with primary antibody for 1 h at room temperature, washed three times for 5 min with PBST, and incubated with secondary antibody for 30 min at room temperature. The primary and secondary antibodies were diluted in PBST and 1% of normal serum of the species from which the secondary antibody is derived. As primary antibodies we used the affinity-purified polyclonal antibodies C44 (1:50 dilution), RA2009 (1:100), B39 (1:500), and C90 (1:100) and the monoclonal antibodies P5D4 (anti-VSV, 1:1000) and M5 (anti-FLAG, 1:100). For co-localization studies, the following monoclonal antibodies were used: 4G3 (anti-U2-B''), 9A9 (anti-U1-A/U2-B'') (44), Y12 (anti-Sm) (45), anti-SC35 (46), and 5E10 (anti-PML) (47). As secondary antibodies, we used FITC-conjugated swine anti-rabbit (1:100 dilution) or rabbit anti-mouse (1:100 dilution) IgG (Dako), and for double immunofluorescence staining, Texas Red-conjugated Goat anti-mouse IgG (1:300 dilution) (Jackson Immunoresearch). After washing three times with PBST, the coverslips were counterstained with 0.1 µg/ml of DAPI (Serva) in PBS for 15 min, washed two times with PBS, dehydrated with water and methanol and mounted in Mowiol (Hoechst). Slides were visualized under a Zeiss Axiophot epifluorescence microscope equipped with appropriate filters and digital images were recorded using a Photometrics high-performance

CH250/A cooled CCD-camera interfaced onto a Macintosh Quadra 950 computer. The images were displayed in red-green-blue pseudocolors on the computer screen using the image analysis and processing software program BDS-image (Biological Detection Systems). Confocal laser-scanning microscopy was performed using a Bio-Rad MRC1000 Laser Scanning microscope. Digitalized images were processed using the Adobe Photoshop software package.

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ABBREVIATIONS

GST, glutathione-S-transferase; KRAB, Krüppel-associated box; NLS, nuclear localization signal; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonyl fluoride; RT-PCR, reverse transcription–polymerase chain reaction; VSV, vesicular stomatitis viral.

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